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What makes resistance to methicillin heterogeneous?

The recent emergence of community-acquired methicillin-resistant *Staphylococcus aureus* (cMRSA) has renewed interest in the mechanisms of methicillin resistance (Okuma *et al.*, 2002). Some cMRSA may have quite low methicillin MICs, comparable to those of susceptible strains (oxacillin MIC $\leq 2 \mu\text{g ml}^{-1}$), which makes them difficult to detect and to distinguish from penicillinase-overproducing strains, also known as borderline methicillin-resistant *S. aureus* (BORSA).

Methicillin resistance is due to the acquisition of a large DNA element, termed staphylococcal cassette chromosome *mec* (SCC*mec*, formerly also called *mec* determinant), which integrates site- and orientation-specifically into the *S. aureus* chromosome. SCC*mec* is considered to be a novel type of mobile element, and has been termed a 'resistance island' by analogy to pathogenicity islands (Ito *et al.*, 1999). The prerequisite for methicillin resistance located on SCC*mec* is *mecA*, which encodes a low-affinity penicillin-binding protein, PBP2' (synonym PBP2a).

A characteristic of methicillin resistance is its usually heterogeneous expression, which means that growth in the presence of β -lactams selects highly resistant subclones from an MRSA population with low methicillin MICs. The frequency at which highly resistant subclones arise is a reproducible, strain-specific characteristic (Tomasz *et al.*, 1991) and usually lies clearly above the rate of spontaneous mutation, but is not likely a mutator phenotype (Finan *et al.*, 2002). With few exceptions (de Lencastre *et al.*, 1993), once high level resistance has been selected, it remains high (Finan *et al.*, 2002). The mechanism leading to formation of these highly resistant subclones is an intriguing problem that remains to be solved.

In an effort to elucidate the causes of heteroresistance, a number of chromosomal factors were characterized whose activity affects the level of resistance. Many of these genes are involved in cell wall biosynthesis and their study has given valuable insight

into this pathway (reviewed by Berger-Bächi & Rohrer, 2002). However, none of these so-called *fem* or *aux* factors has been shown to be the central effector of heteroresistance. Of the few genetic factors whose alteration is able to raise the level of methicillin resistance *in vitro*, none have clearly been shown to be important in clinical isolates.

In this editorial, we will address aspects of heterogeneous resistance to methicillin that may not have received the necessary attention, and discuss a number of new methods that may provide clues on how heteroresistance arises.

Influence of autolytic activity

The major autolysin Atl has been implicated in lytic death in the presence of penicillin as well as in cell separation under normal growth conditions (Sugai, 1997; Sugai *et al.*, 1997). On treatment with penicillin, the staphylococcal cell wall is punctured by the autolytic enzymes of the splitting system, concentrated in so-called murosomes. The internal osmotic pressure leads to loss of cytoplasmic material, which is thought to be the cause of cell death. The cells then disintegrate by generalized autolysis (Giesbrecht *et al.*, 1998).

Since autolysins are clearly the cause of death under the influence of β -lactams, as might be expected, alterations in their activity have been shown in numerous works to affect the level of methicillin resistance. Homogeneous, highly resistant clones have been shown to have reduced autolytic activity (Gustafson & Wilkinson, 1989). A protective effect of NaCl on the susceptible subpopulation was observed when cells were exposed to nafcillin, which may be due to the inhibition of autolysins by high salt concentrations (Chambers & Hackbarth, 1987). While it has been shown that mutation of the *abcA* gene, which is methicillin-inducible (Schrader-Fischer & Berger-Bächi, 2001), leads to increased autolysis and methicillin resistance due to enhanced production of PBP4 (Domanski & Bayles, 1995; Domanski *et al.*, 1997), the actual function of this gene is so far unclear.

The second major cell wall constituent besides peptidoglycan, the teichoic acids, appear to play a crucial role in staphylococcal resistance against methicillin. Mutation of the *llm* gene (similar to the teichoic acid linkage unit synthesis gene *tagO* from *Bacillus subtilis*) reduces methicillin resistance and increases the rate of autolysis (Maki *et al.*, 1994). A reduced degree of teichoic acid D-alanine substitution leads to reduced autolytic activity and, concomitantly, the methicillin MIC rises (O'Brien *et al.*, 1995). The reduction of D-alanine esterification of teichoic acids has a negative effect on vancomycin tolerance, on the other hand (Peschel *et al.*, 2000), which may be an indication to the observed 'incompatibility' of vancomycin and methicillin resistance in some *in vitro* generated mutants (Brandenberger *et al.*, 2000; Sieradzki & Tomasz, 1997).

Taken together, the influence of autolysins on the level of resistance in MRSA should be investigated more closely, as there are some inconclusive results.

Regulatory networks

Methicillin resistance levels depend strongly on external conditions such as temperature, osmolarity, availability of divalent cations, oxygen pressure and light (Matthews & Stewart, 1984), which suggests that specific regulatory systems control the resistance mechanism. A dose-dependent reduction of autolytic enzymes in tolerant strains exposed to methicillin (Goessens *et al.*, 1986) suggested that there is a trigger or regulatory mechanism that controls their production. A regulatory system (*lytSR-lrgAB*) that suppresses autolysin production has indeed been postulated; inactivation of *lrgAB* increased the sensitivity of mutants to penicillin-induced lysis (Groicher *et al.*, 2000).

The global regulators *agr* and *sar* and the alternative transcription factor SigB have been shown to affect methicillin resistance levels; however, the complexity of these intertwined regulatory systems is high and it is not clear how they regulate the

heterogeneous resistance phenotype. It is conceivable that additional levels of regulation exist in *S. aureus* that specifically control heteroresistance.

Cross-talk with β -lactamase regulation has been described in low-level MRSA carrying β -lactamase plasmids. *mecA*, which in most strains is associated with the *mecI* and *mecR1* regulatory genes (Hiramatsu *et al.*, 1992), can be strongly repressed by the β -lactamase repressor (McKinney *et al.*, 2001), which may make such strains appear phenotypically susceptible (Boyce *et al.*, 1990; Hackbarth *et al.*, 1994).

Role of transposable elements in variations of gene expression

Another conceivable mechanism for creation of highly resistant subclones would be the transposition of mobile genetic elements such as transposons or insertion sequences, leading to the alteration of transcriptional activity in the target regions of the chromosome.

Several variants of a hybrid promoter formed by transposition of IS256 into the 5' region of *llm* were shown to increase both *llm* transcription and methicillin resistance to distinct levels (Maki & Murakami, 1997). IS257, which is present on SCC*mec*, has also been shown to alter the transcriptional activity of neighbouring genes (Simpson *et al.*, 2000). A type of phase variation caused by IS256 insertion-mediated gene disruption has been described for the regulation of exopolysaccharide synthesis in *Staphylococcus epidermidis* (Ziebuhr *et al.*, 1999). It is possible that gene rearrangements, i.e. duplications or deletions caused by IS elements, could lead to stable genetic alterations in heterogeneously methicillin-resistant *S. aureus*.

New approaches that may shed light on heteroresistance

Global studies into the alterations that highly methicillin-resistant *S. aureus* clones undergo are rare so far, whether on the genomic, transcriptional or proteomic level. The completion of the genome sequence is one important step towards understanding the changes leading to high resistance levels. A first approach to globally comparing such strains with their parents may be DNA–DNA hybridization on DNA microarrays to show possible mutational changes on the

DNA level. As it is likely that a global regulator plays a role in heterogeneous resistance, its activation or suppression would lead to the pleiotropic effects that appear to allow for highly resistant subclones. Such a mechanism could be addressed by analysing the transcriptional patterns of MRSA on DNA microarrays. The top level of comparing low-level resistant parents with their highly resistant subclones is analysis of the proteome. Two-dimensional protein gels yield a type of snapshot of the state of different strains. There has so far been one study that analysed the proteins induced by cell-wall-active antibiotics (Singh *et al.*, 2001). With the data gained in such experiments, along with increasing knowledge about the activities of global regulatory networks, it should be possible to finally pinpoint the trigger that enables the segregation of highly methicillin-resistant subclones on selection with methicillin.

Conclusions

At this stage, it can be summarized that although the *mecA* gene is the prerequisite for methicillin resistance, a continuum of resistance levels exists, the regulation of which has not been elucidated so far. Since this heterogeneous phenotype of methicillin resistance is possibly an effect of a pleiotropic regulatory mechanism, only recently developed techniques that allow the monitoring of cellular activity on a global scale may shed light on its identity.

It is clear that new antimicrobial agents against staphylococci will be urgently required, especially with the prospect of highly vancomycin-resistant *S. aureus* (VRSA) carrying *vanA*-type resistance genes emerging (CDC, 2002), and the elucidation of the origins of heteroresistance to methicillin may contribute to the discovery of new targets for such medicines.

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